Insect Molecular Biology (2003) **12**(3), 271–280

Quantitative expression analysis of a *Glyptapanteles indiensis* polydnavirus protein tyrosine phosphatase gene in its natural lepidopteran host, *Lymantria dispar*

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Abstract

In the present study, expression of a newly identified *Glyptapanteles indiensis* polydnavirus (GiPDV) gene encoding a putative protein tyrosine phosphatase (PDVPTP) was monitored *in vivo* in the parasitized host, *L. dispar*, using one step RT-PCR. Expression levels of the PDVPTP transcript were also evaluated in various host tissues at different times post parasitization (pp) using RT quantitative competitive PCR (RT-qcPCR). Expression levels varied, with the most abundant transcript detected in host haemolymph 2 h pp. The high expression level in host haemolymph at an early stage of parasitization suggested a potential role for viral PDVPTP in disruption of the host immune system and protection of the endoparasitoid egg from encapsulation. Additionally, the PDVPTP gene or its homolog(s) mapped to more than one GiPDV genomic DNA segment, which may account for its increased level of expression in the absence of virus replication.

Keywords: *Glyptapanteles indiensis*, *Lymantria dispar*, PDV, Gene expression, RT-PCR, RT-qcPCR.

Introduction

Polydnaviruses have been found in parasitic Hymenoptera in the families of Braconidae and Ichneumonidae. These viruses have polydisperse genomes that consist of double stranded, covalently closed superhelical DNAs varying in size and molar ratio. The viruses replicate in the calyx epithelial cells of wasp oviduct, and virions are secreted into the lumen of the oviduct where they accumulate along with the wasp eggs and a complex of oviduct proteins. During oviposition, calyx fluid is injected into the host larva, along with parasite eggs. Within host tissues, polydnaviruses do not replicate but many viral genes are expressed. Edson *et al*. (1981) demonstrated that the *Campoletis sonorensis* polydnavirus (CsPDV) was involved in the protection of the parasite's eggs from the host's immune response. In their experiments, parasitic eggs injected without virus into *Heliothis virescens* larvae were encapsulated, while eggs coinjected with virus were not encapsulated and developed normally. Subsequent studies in other parasite-host systems showed that suppression of the encapsulation response in the host was due to alterations of haemocytes which are essential for the immune response against foreign objects, such as parasitic eggs. It is believed that polydnavirus gene products exert multiple effects in suppression of the host immune system, including alteration of haemocyte behaviour, reduction of haemocyte population, and inhibition of haemolymph phenoloxidase activity (Edson *et al*., 1981; Davies *et al*., 1987; Strand & Pech, 1995; Summers & Dib-Hajj, 1995; Lavine & Beckage, 1996). Other significant physiological effects, such as developmental arrest, hormone imbalance, and growth inhibition are also believed to be associated with polydnavirus expression (Vinson *et al*., 1979; Beckage, 1985; Davies *et al*., 1987; Dover *et al*., 1987, 1988; Tanaka *et al*., 1987; Yamanaka *et al*., 1996).

The effects of polydnavirus in suppressing immune response and altering growth and development to ensure successful development of the parasite in the host insect have prompted a number of studies on the expression of polydnavirus genes in parasitized hosts. The Ichnovirus, CsPDV, has been the most extensively studied polydnavirus at the molecular level (Fleming *et al*., 1983; Blissard *et al*., 1986a, 1986b; Theilmann & Summers, 1986, 1988; Dib-Hajj *et al*., 1993; Li & Webb, 1994; Cui & Webb, 1996). Fleming *et al*. (1983) provided the first evidence that CsPDV genes are transcribed in parasitized *H. virescens* larvae. By using labelled CsPDV genomic DNA as a probe, CsPDV transcripts could be detected in *H. virescens* larvae as early as two hours post parasitization (pp) and continued to be detected for 9 days while *C. sonorensis* larvae developed endoparasitically. Blissard *et al*. (1986a,b) reported

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at least 12 different sized CsPDV mRNAs detected by Northern analysis in parasitized *H. virescens* during the course of endoparasite development. Viral genes expressed in the lepidopteran host have been also described from the bracoviruses of *Cotesia congregata* (Harwood *et al*., 1994), *C. rubecula* (Asgari *et al*., 1996), *C. karyai* (Yamanaka *et al*., 1996) and *Microplitis demolitor* (Strand *et al*., 1997).

Glyptapanteles indiensis is a braconid wasp that parasitizes the gypsy moth, *Lymantria dispar. G. indiensis* PDV (GiPDV) is essential for successful parasitism. To understand the mechanism of GiPDV in suppression of the host immune system and protection of the developing endoparasite, temporal GiPDV gene expression during parasitism and levels of GiPDV gene expression in the parasitized host need to be measured comprehensively. However, knowledge of GiPDV gene expression in the parasitized host is limited compared with that for other polydnaviruses. GiPDV gene transcriptional activity in different tissues of parasitized hosts has not been studied.

The classical Northern-blot technique for measuring gene expression has many limitations such as poor sensitivity with low-abundance mRNAs, the requirement for considerable tissue, and the time required to obtain results. To overcome these limitations, more modern approaches have been employed. RT-PCR is a highly sensitive method for detecting the presence or absence of specific transcripts from small quantities of RNA and can represent a valuable replacement for Northern analysis, *in situ* hybridization, or other immunoassays. The development of the RT-quantitative competitive PCR (RT-qcPCR) method has remarkably improved the accuracy of RT-PCR. By using an internal standard (IS) which is added to the system following the RT prior to PCR and competes for the same sequence-specific primer pairs with target cDNA in the same reaction tube, RT-PCR has become a quantitative method not only for the detection but also for the absolute quantification of mRNA transcripts (Zimmermann & Mannhalter, 1996).

Previous studies in our laboratory reported that GiPDV is integrated not only as a provirus within the parasitoid wasp genome, but also *in vitro* within the chromosomal DNA of virus infected cells derived from the natural host of the parasitoid, *L. dispar* (Gundersen-Rindal & Dougherty, 2000). Recently, within the GiPDV genome, including the circular genome segment associated with integration, a family of viral genes encoding putative protein tyrosine phosphatases (PTPases) was identified (D. Gundersen-Rindal, unpublished results). In the present study, one of these putative PTPases, designated PDVPTP (GenBank accession no. AF453875), was mapped to the GiPDV by field inversion gel electrophoresis and Southern hybridization. *In vivo* expression of PDVPTP at various times in the parasitized *L. dispar* host was evaluated using one step RT-PCR. Further, the expression levels of PDVPTP in different host tissues and at different times pp were quantified using an RT-qcPCR assay.

Results

In order to map PDVPTP to the GiPDV genome, GiPDV genomic DNAs were size fractionated by pulse field certified agarose gel. Separated genomic DNA segments were transferred from the gel to membrane, and hybridized with DIG-labelled PDVPTP. PDVPTP hybridized to two genomic DNA segments (F and G) as shown in Fig. 1.

In order to identify the expression of viral PDVPTP transcript in the parasitized *L. dispar* larvae and the time course of expression during parasitism, total RNA was isolated from host larvae at various times (2 h, 6 h, 12 h, 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, 16 days and 18 days) pp by *G. indiensis*, and conventional one step RT-PCR was performed. As shown in Fig. 2A, PDVPTP was expressed in the parasitized host larvae. The PDVPTP transcript was detected in parasitized *L. dispar* larvae as early as 2 h pp and the continuous expression was detected up to 8 days pp. No detectable level of expression was observed 10 days after parasitization. No PDVPTP transcript was present in RNAs isolated from nonparasitized *L. dispar* larvae. Also, the relative quantities of viral transcripts expressed in the host larvae varied with time of parasitism. Varying levels of gene expression were seen in parasitized host larvae. The highest level of expression occurred 2 h pp as indicated by greatest band intensity, but thereafter started to decline. The level of PDVPTP expression reached a steady stage from 4 h to 6 days pp and no significant fluctuations were observed during this period of time. At day 8 pp, expression declined again as indicated by a significantly reduced band intensity. No expression was observed from the RNA of nonparasitized host larvae. All RNA samples were found to be free of DNA contamination (Fig. 2B).

To examine the expression levels of PDVPTP in different host tissues, *L. dispar* larvae at 2 h, 1 day, 4 days and 8 days pp were dissected and host tissues of haemolymph, brain, and fat body were collected individually for RT-qcPCR analysis. As conventional RT-PCR indicated expression levels in the parasitized host 4 h to 6 days pp were similar, host tissues were collected only at 1 day and 4 days during this period. RNA templates isolated from each tissue were subjected to RT-qcPCR amplification. Seven 10-fold dilutions of competitive IS (10^7 to 10 copies) were coamplified individually with a constant amount of first strand target cDNA in the same tube. The RT-qcPCR amplification resulted in two different sized bands, the upper bands representing the amplification products of target cDNA (ptp), and the lower bands representing the amplification products of IS (D-ptp). A series of decreasing

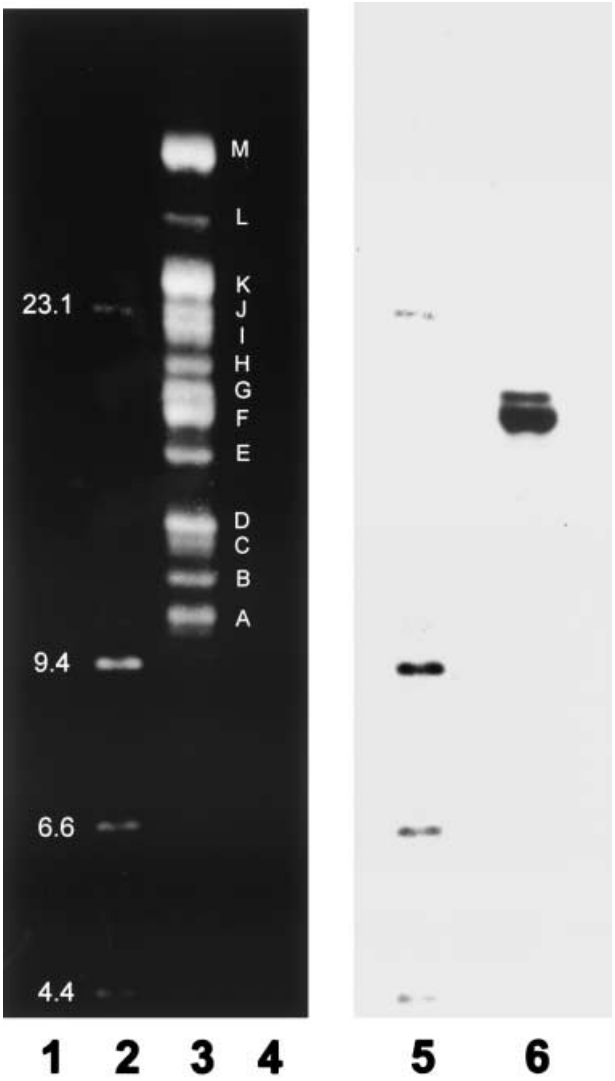


Figure 1. Localization of PDVPTP in the GiPDV genome. DIG-labelled linear λ DNA markers digested with *Hin*dIII (lane2) and undigested GiPDV genomic DNA (lane 3) were loaded into a 0.8% pulse field certified agarose gel. Field inversion gel electrophoresis was performed at 4 °C for 48 h. The λ DNA marker sizes are given in lane 1. The major DNA segments are indicated by uppercase letters. Separated GiPDV DNA segments were blotted on to membrane and probed with DIG-labelled PDVPTP DNA, which hybridized to two genomic DNA segments as shown in lane 6.

amounts of IS (from 10^7 copies to 10 copies) resulted in a gradual decrease of the amplification products for IS and a gradual increase of the amplification products for target cDNA (Fig. 3A). Individual gel bands were quantified by densitometric analysis. After correcting for PCR product size of competitive IS (D-ptp), the copy numbers of the IS, D-ptp (*x*-axis), were plotted against the ratio of the corrected D-ptp band intensity value divided by ptp band intensity value. The point where the ratio of the corrected D-ptp bands intensity value/ptp band intensity value equaled 1 was the point of equivalence of the two templates and represented the amount of target cDNA present in the tissues (Fig. 3B). As shown in Figs 3 and 4, PDVPTP was expressed in the tissues of larval host haemolymph, brain, and fat body, but the expression levels varied in each tissue tested. The highest level of expression was seen in haemolymph. Moderate expression was observed in the brain and weakest expression was observed in fat body of host. Further, expression levels varied at different times pp. In the haemolymph, the highest level of expression was detected at 2 h pp, where the point of equivalence was 600 copies per 0.5 μg RNA (SD = 79.25, $N = 3$). Moderate expression was observed at 1 day and 4 days pp, where the points of equivalence were 280 copies (SD = 64.46, $N = 3$) and 230 copies (SD = 38.16, $N = 3$) per 0.5 μg RNA, respectively. The weakest expression was observed at 8 days pp, where the point of equivalence was 130 copies per 0.5 μg RNA (SD = 16.11, $N = 3$). In the brain, the highest level of expression was also seen at 2 h pp, where the points of equivalence were 170 copies per 0.5 μg RNA (SD = 14.75, $N = 3$). Moderate expression was observed at 1 day and 4 days pp, where the points of equivalence were 45 (SD = 5.32, $N = 3$) and 42 (SD = 4.02, $N = 3$) copies per 0.5 μg RNA, respectively. No significant difference in the level of expression was observed in the brain 1 day and 4 days pp. No detectable expression was observed in the brain 8 days pp. In the fat body, the only time with significant expression of PDVPTP was 2 h pp, where the points of equivalence were 120 (SD = 22.13, $N = 3$) copies per 0.5 μg RNA. The expression of PDVPTP in fat body at 1 day, 4 days, and 8 days pp were below the lower limit of IS (10 copies per 0.5 μg RNA) therefore the standard curves for

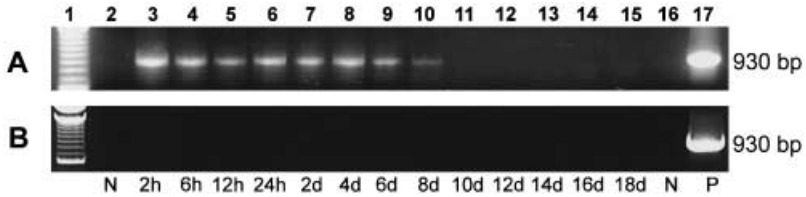


Figure 2. Time course expression in *L. dispar* larvae parasitized by *G. indiensis*. Total RNA was extracted from *L. dispar* larvae at various times pp. All RNA samples were treated with DNAse and then subjected to RT-PCR analysis (A). The absence of contaminating DNA in the RNA samples was verified by running PCR directly without reverse transcription (B). A and B lane labels are identical: Lane1, 100 bp DNA ladder; lane 2, RNA from nonparasitized larvae; lanes 3–15, RNA extracted from parasitized larvae at 2 h, 6 h, 12 h, 24 h, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, 16 days, and 18 days pp, respectively; lane 16, negative control (without template); lane 17, positive control (previously identified PDVPTP positive sample).

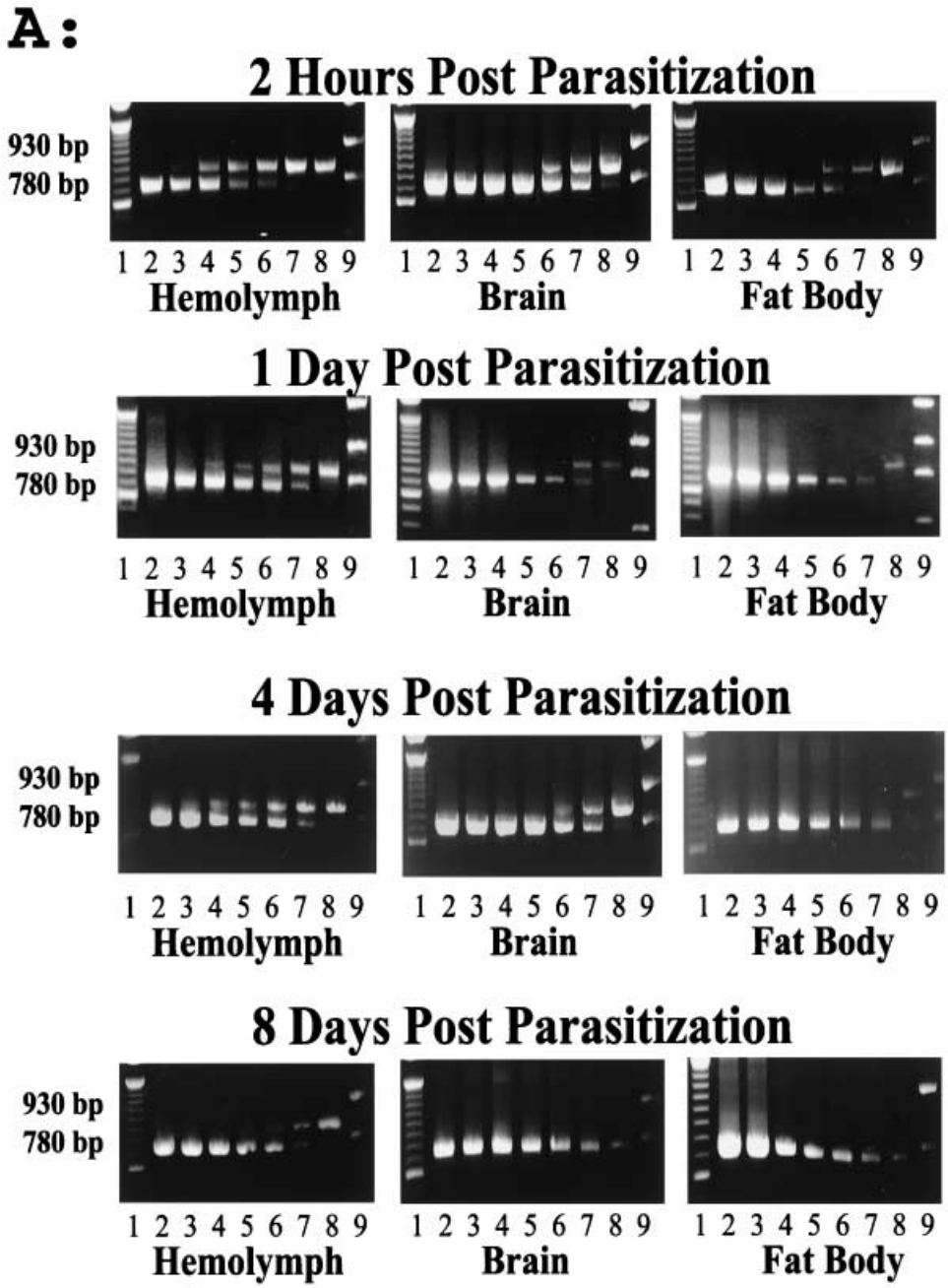


Figure 3. (A) Quantification of PDVPTP gene expression in host tissues of haemolymph, brain, and fat body 2 h, 1 day, 4 days, and 8 days pp. The ethidium bromide stained gels of RT-qcPCR assays are shown. Parasitized *L. dispar* larvae were dissected and host tissues haemolymph, brain, and fat body were collected individually. RNA templates isolated from each tissue were subjected to RT-qcPCR amplification. Seven 10-fold dilutions of competitive IS (10^7 to 10 copies) were coamplified individually with a constant amount of first strand target cDNA in the same tube. The ethidium bromide stained gels of the RT-qcPCR assay are shown. The positions of the expected product of target, PDVPTP (930 bp) and IS, D-ptp (780 bp) are indicated. Lane 1, 100 bp DNA ladder. Lanes 2–8, 10^7–10 copies of IS. Lane 9, low DNA mass ladder. (B) Standard curves for PDVPTP gene RT-qcPCR 2 h, 1 day, 4 day, and 8 days pp. The copy number of IS is plotted against the ratio of the band intensities of corrected IS (D-ptp) divided by target template (ptp). The point where the ratio of the corrected D-ptp bands intensity values/ptp band intensity values equals 1 was the point of equivalence of the two templates and represents the amount of the target cDNA present in the tissues. Because the expression of PDVPTP in fat body at 1, 4, and 8 days pp was below the low limit of IS (10 copies per 0.5 μg RNA), a standard curve was unable to be established.

expression in fat body at 1 day, 4 days, and 8 days pp were unable to be established (Fig. 3B).

During the development of this RT-qcPCR method, preliminary studies were conducted to verify the integrity of the RNA sample, to optimize the assay conditions, and to set up the lower limit for competitive IS. In order to verify that RNA preparations were free of DNA contamination, all RNA samples were subjected to one

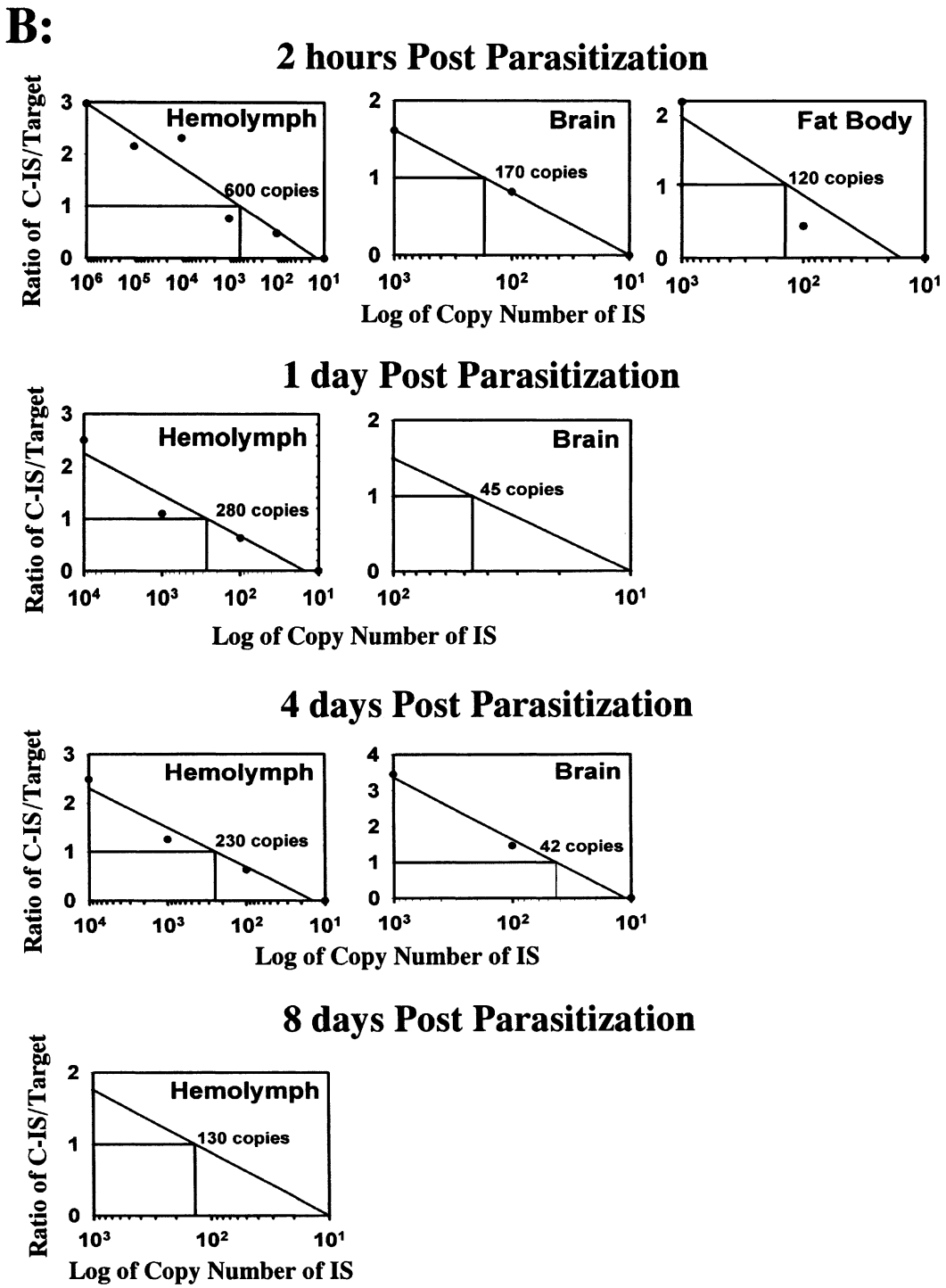


Figure 3. (*continued*)

step RT-PCR in the absence of reverse transcriptase. The results indicated that all the RNA samples were DNA free after DNase I treatment. The determination of optimal conditions for qcPCR included identification of optimized oligonucleotide primer and $MgCl_2$ concentrations, and the choice of AmpliTaq Stoffel Fragment, which was critical for multiplex PCR. To evaluate the amplification efficiency and sensitivity of competitive internal standard, 10-fold dilutions of qcIS from 10^9 to 1 copy were prepared and added to the PCR mixture. The lower limit of detection for the IS using in this method was approximately 10 copies.

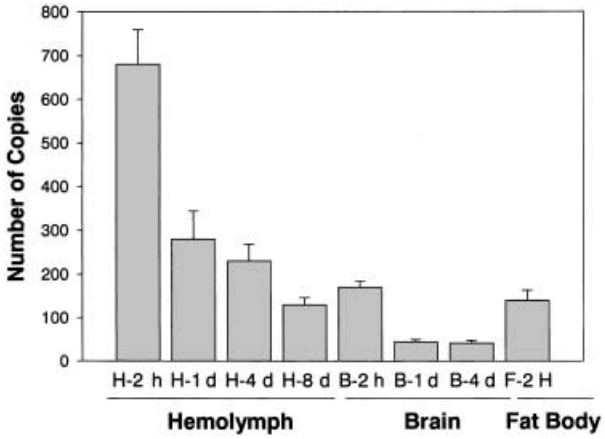


Figure 4. Comparison of PDVPTP gene expression levels as indicated by the number of transcripts present in the parasitized host tissues haemolymph (H), Brain (B) and fat body (F) at different times (2 h, 1 day, 4 days, 8 days) pp. PDVPTP was preferentially expressed in the haemolymph. At 2 h pp, transcript copies were 3.5–5 times higher in the haemolymph than in brain and fat body. At 1 day and 4 days pp, transcripts in the haemolymph were six times higher than in brain. During this period of time, expression in fat body was too weak to establish a standard curve for measuring transcript copies. At 8 days pp, transcript was only detected in the haemolymph.

Discussion

RT-qcPCR is a sensitive, accurate and specific method for detecting and quantifying gene expression in *L. dispar* larvae parasitized by *G. indiensis*. RT-qcPCR is advantageous over other available methods such as Northern blot, *in situ* hybridization, and the ribonuclease protection assay. The superiority of this method lies in the fact that RT-PCR is capable of detection of a very low copy number mRNA in a considerable background of endogenous RNA. Moreover, the same set of primers compete for amplification of both unknown target template and competitive template, therefore the target and the competitor are subject to identical amplification conditions and the variables affecting amplification have the same effects on both. Finally, because the ratios between the target template and a dilution series of qcIS remain constant during extensive PCR cycling, the relative amounts of target cDNA vs. qcIS over many cycles of amplification can be quantified by densitometry and the initial concentration of the target cDNA can be calculated. Because of its high sensitivity, accuracy, and specificity, the RT-qcPCR method applied to quantify PDVPTP gene expression in parasitized *L. dispar* larvae described in this paper can be adopted easily to any gene of interest in future polydnavirus–parasite–host interaction studies, when a popularly used real-time quantitative PCR machine is not accessible.

Investigation of the time-course expression of PDVPTP in parasitized *L. dispar* larvae indicated that it was differentially expressed. Transcript could be detected in the host as early as 2 h and continued for 8 days after parasitization. No detectable level of expression was observed 10 days pp. In an earlier study, Blissard *et al*. (1986a,b) detected more than 12 CsPDV transcripts in parasitized *Heliothis virescens* by 2 h pp. Transcripts were most abundant 24–48 h pp and were present in the host for 8 days continuously. Similarly, Theilmann & Summers (1988) showed CsPDV genes were expressed most abundantly 2–6 h pp and declined at later time points. The initial expression of PDVPTP in parasitized *L. dispar* larvae appeared to be in line with CsPDV expression in *H. virescens*. Peak expression of PDVPTP was observed 2 h pp, then declined until eight days pp. No transcript was detected thereafter. Because PDVPTP was expressed in the parasitized host and transcript was most abundant at an early stage of parasitization (2 h pp), the gene product may be involved in the disruption of the host immune system and protection of the endoparasitoid egg from encapsulation. The persistence of PDVPTP transcript in the parasitized host may indicate a corresponding function in which product is required immediately in host immunosupression and also continuously in endoparasitoid development. In 1981, Edson *et al*. first reported that PDV-induced factors were causal agents in the suppression of host encapsulation and alteration of host haemocyte behaviour. Previous studies on the timing of immune events in the parasitized host showed that CsPDV mRNAs and their corresponding proteins mediated encapsulation beginning at 30 min through to 24 h pp and immunosuppressive activities were sustained for 5 days continuously pp (Luckhart & Webb, 1996). As the major function of polydnavirus in the host is to protect the parasitoid egg from encapsulation, most polydnavirus transcripts reported to date were seen shortly after parasitization and appeared to be related to the immunosuppressive function of the polydnaviruses.

Expression kinetics of polydnavirus genes can be an indicator of the immunosuppressive activity in the host. Analysis of PDVPTP tissue-specific expression showed preferential expression in the haemolymph. At 2 h pp, transcripts were 3.5–5 times more abundant in haemolymph than in brain or fat body. At 1 day and 4 days pp, transcripts in haemolymph were six times more abundant than in brain. During this period of time, expression in fat body was too low to establish a standard curve for measuring copies of transcript. At 8 days pp, PDVPTP transcript was detected in the haemolymph exclusively (Fig. 4). In a separate study, a different, unrelated GiPDV host-specific viral gene was also found to be expressed preferentially in the host haemolymph less than 1 h pp (Y. P. Chen *et al*., unpublished results). It is unknown whether these GiPDV genes have any functional relationship. The differential expression of polydnavirus genes in host tissues has been reported for several wasp species (Blissard *et al*., 1986a,b; Harwood *et al*., 1994; Asgari *et al*., 1996; Yamanaka *et al*., 1996; Strand

et al., 1992; Strand *et al*., 1997). When polydnavirus is injected into the host with eggs during oviposition, virions penetrate into host tissues and gene expression begins. The gene encoded products have been shown to cause removal of haemocytes from circulation and disruption of their spreading behaviour. This results in failure of haemocytes to spread on a foreign substance and the loss of ability to encapsulate parasitoid eggs, ultimately resulting in hatch and successful development of parasitoid eggs (Edson *et al*., 1981; Davies *et al*., 1987; Strand & Pech, 1995; Summers & Dib-Hajj, 1995; Lavine & Beckage, 1996). The preferential expression in host haemolymph, together with the finding that the most abundant expression occurred at an early stage of parasitization (2 h pp), suggests a role for the PDVPTP-encoded product in the disruption of the host immune system or protection of the endoparasitoid egg from encapsulation. The expression of PDVPTP in host brain suggests that it may also be involved in inhibition or slowing of host development. The inhibition of host development and reproduction is often the result of disruption of the host endocrine system. Pennacchio *et al*. (1998a,b) examined the effects of *C. nigriceps* polydnavirus on inactivation of the host prothoracic gland and regulation of host protein synthesis. They demonstrated that polydnavirus-induced alteration of host protein synthesis and ecdysteroidogenesis was correlated to the depression of host protein kinase activity and inhibition of regulatory target protein phosphorylation. The potentially significant role for PDVPTP in regulating host protein synthesis by negatively modulating the level of kinase phosphorylation is currently being investigated through *in vitro* functional assays in our laboratory. Polydnavirus induced alteration of host development and reproduction has been documented in the naturally parasitized or viral injected larvae of *H. virenscens, Manduca sexta*, and *C. inanitus* (Tanaka & Vinson, 1991; Dushay & Beckage, 1993; Beckage *et al*., 1994; Johner & Lanzrein, 2002). The infection and expression of PDVPTP in brain tissue is further corroborated with previous findings that expression in the host could interfere with signal transduction of hormones and disrupt developmental activity of insects. Because fat body is the major site of protein, lipid, and carbohydrate synthesis and storage, it is immersed in haemolymph to facilitate the exchange of metabolites. Limited expression of PDVPTP in fat body may suggest its involvement in the regulation of host physiological activity. However, fat body could have been contaminated with haemolymph and additional studies are needed to confirm this.

It is interesting to note that the PDVPTP gene hybridized to two different GiPDV genomic segments, it's presumed segment of origin, F, and one other, G. This suggested homologous sequences between the DNA segments, or nested segments, as described in the CsPDV ichnovirus system (Cui & Webb, 1997). It has been suggested that segment nesting may function to increase gene copy number and enhance the level of essential gene expression in the absence of viral replication (Xu & Stoltz, 1993; Cui & Webb, 1997). It is possible that the PDVPTP gene or its homolog(s), coded on different genomic segments, could be expressed simultaneously to multiply the function of PDVPTP-encoded protein in the host. Although our understanding of GiPDV gene expression in the parasitized host is limited, it seems likely that multiple polydnavirus genes, including the PTPases, expressed differentially in host haemolymph, brain, and fat body, serve important roles in host regulation.

Experimental procedures

Insect rearing and parasitization

G. indiensis was imported from India for culture at the USDA-ARS, Beneficial Insects Introduction Laboratory, Newark, DE and fed on 30% honey water. Host larvae (*L. dispar*) were reared on a high wheat-germ diet in 200 ml cups with paper lids. Both wasp and host larvae were maintained at 26 °C, 50% RH, and a 16L:8D photoperiod according to the protocol established by Bell *et al*. (1981).

Parasitization of gypsy moths by *G. indiensis* was conducted by exposing twenty-five late first instar gypsy moth larvae to two *G. indiensis* females for 24 h in an untreated paper carton (473 ml) with a plastic cover, with a source of high wheat-germ diet, water-soaked cotton wick, and small streaks of honey on the lid for feeding parasitoids. Following a 24-h exposure period, adult parasitoids were removed and parasitized gypsy moth larvae were reared until cocoons were formed from parasitized hosts. Cocoons were then removed and stored at 24 °C for adult parasitoid emergence.

For time course experiments, parasitization of gypsy moth was conducted by exposing an individual gypsy moth larva to a *G. indiensis* female within a 35 × 10 mm Petri dish to ensure 100% parasitization. After a single oviposition was observed, the parasitized larva was removed from the Petri dish and put into a plastic cup with high wheat-germ diet. Post parasitization times were calculated and recorded from the initiation of parasitization.

Virus purification and viral DNA isolation

GiPDV was purified from the calyx fluid of female *G. indiensis* as described by Beckage *et al*. (1994). Briefly, calyx fluid was collected by dissecting ovaries of female wasps in cold phosphate-buffered saline (PBS) solution. The calyx fluid was filtered through a 0.45-μm filter to remove the eggs and cell debris. The resulting calyx fluids were incubated in an equal volume of DNA extraction buffer (4% sarcosyl, 1% SDS, 50 mM EDTA, 10 mM Tris, 0.2 M NaCl, 20 μg/ml RNase, 50 μg/ml proteinase K) at 50 °C for 2 h, followed by two extractions with buffer-saturated phenol (Gibco BRL Life Technologies). DNA was precipitated in 2 volumes of cold ethanol and resuspended in TE buffer.

Field inversion gel electrophoresis and Southern hybridization

Undigested GiPDV genomic DNA and a molecular size standard (digoxigenin-labelled linear λ DNA digested with *Hin*dIII) were electrophoresed on a 0.8% (wt/vol) pulse field certified agarose gel

as described elsewhere (Gundersen-Rindal & Dougherty, 2000). separated viral genomic DNA segments were transferred from the gel to membrane (Hybond-N+, Amersham Pharmacia Biotech). PCR amplified PDVPTP fragments were digoxigenin (DIG) labelled according to the manufacturer's instructions (Boehringer Mannheim). Membrane with separated GiPDV genomic segments was hybridized to DIG-labelled PDVPTP probe in hybridization solution for 16 h at 50 °C. The membrane was exposed to X-ray film (Amersham) for signal detection.

Extraction of RNA

Parasitized *L. dispar* larvae were frozen in liquid nitrogen and ground as powder for RNA extraction at various time intervals (2 h, 6 h, 12 h, 1 day, 2 d, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, 16 days, 18 days) pp. TRIzol Reagent (RNA extraction kit, Life Technologies) was used for total RNA isolation according to the manufacturer's instructions. After determining the concentrations of RNA samples by measuring the absorbency at 260 nm, the samples were stored at –80 °C for further analysis. The quality of the RNA was analysed by agarose gel electrophoresis in the presence of 1% formaldehyde.

For analysis of PDVPTP gene expression in different tissues, the tissues of host haemolymph, brain and fat body were collect at 2 h, 1 day and 8 days pp. For extraction of RNA from haemolymph, parasitized *L. dispar* larvae were incubated in 50 °C water for 10 minutes to denature any phenoloxidase activity, and then chilled on ice immediately. By gently squeezing the body of the larva, haemolymph was drawn from the larva with a glass capillary tube through an opening made by cutting off a hindleg. After collecting haemolymph, the parasitized *L. dispar* larvae were dissected in phosphate-buffered saline (Life Technologies) under the microscope and the tissues of the brain and fat body were collected separately. To prevent contamination with haemolymph, brain and fat body tissues were rinsed with PBS three times prior to RNA extraction. The procedures of RNA extraction were the same as described above.

Construction of quantitative competitive internal standard

The procedures of constructing the RT-PCR quantitative competitive internal standard (IS) are shown in Fig. 5(A). The IS was created by amplification of PDVPTP DNA fragment (ptp) with a pair of composite primers (ptp-IS-F/ptp-IS-R). The cloning of the GiPDV circular segment p157 (now known as segment F) has been described previously (Gundersen-Rindal & Dougherty, 2000). The composite primers consisted of a 25 base exterior primer (ptp-F1/ptp-R1) starting from the 5′ end of the primer and a 25-base internal primer (ptp-F2/ptp-R2) located 75 bases downstream on the DNA template in relation to the 3′ end of the exterior primer (Fig. 5B), thereby resulting a 150 base deletion in the final PCR product. Except for a 150 bp deletion, the IS contained the same sequence and shared the same primer recognition site as the PDVPTP gene. The amplification was performed by adding 50 ng of p157 plasmid DNA to a mixture containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl; 0.01% gelatin), 0.2 mM each dNTP, 0.5 μM each composite primers, 2.5 U of AmpliTaq Gold (Perkin Elmer). The reaction was conducted for 40 cycles at 94 °C for 30 s, 55 °C for 1min, and 72 °C for 2 min. The 780 bp of deleted ptp fragment (D-ptp) were purified from the gel using a Wizard PCR Preps DNA Purification System (Promega). To facilitate the production of qcIS, the D-ptp fragment was ligated into a pCR 2.1 vector (T.A. Cloning Kit, Invitrogen). The ligated D-ptp plasmid was transformed into DH5a-T1 competent cells (Invitrogen). Plasmids containing the complete DNA sequence of D-ptp were used as an internal control for the RT-qc-PCR. The copy number of IS was calculated for PDVPTP gene based on its molecular weight and concentration.

RT-PCR and RT-qcPCR

All samples were treated with amplification grade DNAse I (Life Technologies) prior to synthesis of the first-strand cDNA. The absence of DNA in the RNA samples was verified by running PCR directly without reverse transcription.

For analysis of the time-course expression of PDVPTP, one step RT-PCR was applied with PDVPTP gene specific primers (ptp-F/ptp-R) (Fig. 5). The Access RT-PCR System (Promega) was used for amplification according to the manufacturer's instructions.

RT quantitative competitive PCR was conducted to quantify PDVPTP gene expression in the tissues of host haemolymph, brain and fat body at 2 h, 1 day, 4 days, and 8 days pp. Reverse transcription was performed in a final volume of 20 μl containing 5 μg of total RNA in the presence of 1× RT buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl, 3 mM $MgCl_2$) mM dNTPs, 1 mM dithiothreitol (DTT), 200 units SUPERSCRIPT™. II RNase H^- reverse transcriptase (Life Technologies), and 2.5 μM oligo(dT) primer. The reaction was incubated at 42 °C for 45 min and then inactivated by heating to 75 °C for 15 min and cooled on ice.

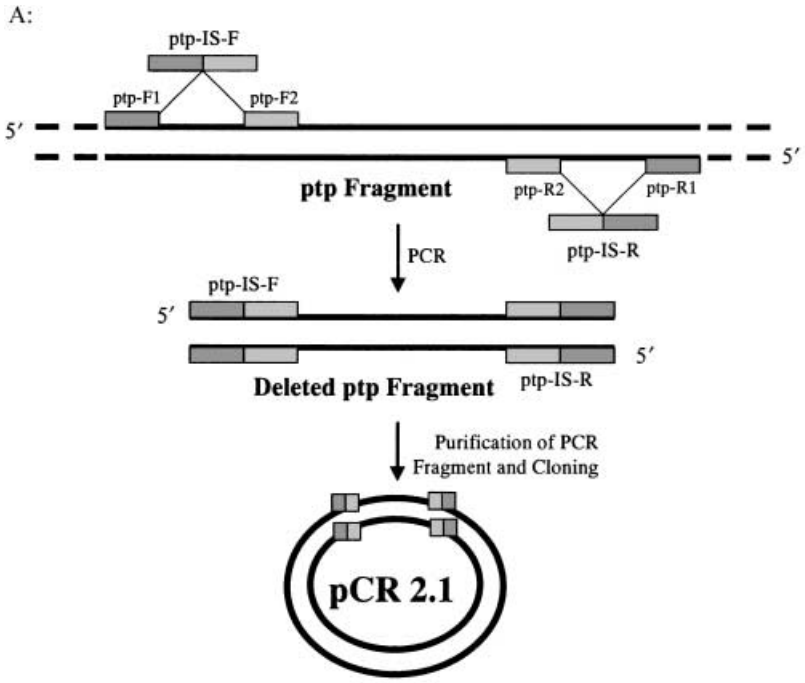
A series of quantitative competitive PCRs were set up using 10-fold dilutions of DNA of the IS, ranging from 10^7 to 10 copies with a constant amount of the first strand of cDNA. PCR was performed in a 50-μl reaction mixture containing 1× Stoffel buffer (10 mM Tris-HCl, pH 8.3; 10 mM KCl; 2.5 mM $MgCl_2$), 0.4 mM each dNTP, 0.5 μM each primers (ptpF1 and ptpR1), and 10 U of AmpliTaq polymerase, Stoffel fragment (Applied Biosystems). The reaction was conducted for the first five cycles at 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and for subsequent 30 cycles at 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 2.5 min plus 5 s per cycle.

Sample analysis and quantification

PCR reaction products were visualized by 1.2% agarose gel electrophoresis in the presence of ethidium bromide. A 100-bp DNA ladder (Life Technologies) and a Low DNA Mass Ladder (Life Technologies) were included as standards on each gel. The intensities of the bands were quantified by densitometry (LabWorks™, Image Acquisition and Analysis Software, UVP). Because the IS had a 150 bp deletion compared with the target cDNA, the reduced length could result in a decrease in the incorporation of ethidium bromide. To resolve this problem, the fluorescence intensity of D-ptp was corrected following a correction equation:

corrected D-ptp band intensity = (original D-ptp band intensity) × (length of ptp <930 bp>)/length of D-ptp <870 bp>

developed by Menzo *et al.* (1992). After correcting the PCR product size of the internal standard, the values were plotted as concentration of the internal standard on the *X*-axis vs. the ratio of the corrected IS band densities to the target cDNA band densities on the *Y*-axis. The ratio between the corrected IS and target values were graphed on a logarithmic scale.



B:

ptp-F1: 5′-ATGCGGATCCATGGGTTGTTACAGTTCAA-3′
ptp-R1: 5′-GACTGAATTCTGCTTACATATAAAACGTTA-3′

ptp-F2: 5′-ACATAATAAGTGAGGAAACAA-3′
ptp-R2: 5′-ATTAACTCTACCCGTCTTTA-3′

ptp-IS-F:
5′-ATGCGGATCCATGGGTTGTTACAGTTCAA*ACATAATAAGTGAGGAAACAA*-3′
ptp-IS-R:
5′-GACTGAATTCTGCTTACATATAAAACGTTA*ATTAACTCTACCCGTCTTTA*-3′

Figure 5. Construction of RT quantatitive competitive-PCR (RT-qcPCR) internal standard. (A) Schematic representation of construction method. The PDVPTP gene was amplified with chimeric primers (D-ptp-F/D-ptp-R) composed of a 25-base exterior primer (ptp-F1/ptp-R1) starting from the 5′ end of the primer and a 25-base internal primer (ptp-F2/ptp-R2) which was 75 bases downstream on the DNA template in relation to exterior primer at the 3′ end of the primer, thereby resulting a 150-base deletion in the final PCR product. The PCR fragments of internal standard (D-ptp) were gel purified and cloned into the pCR 2.1 vector. (B) Sequences of primers pairs used in the construction of RT-qcPCR internal standard (ptp-IS-F/ptp-IS-R) and in the detection and quantification of the viral gene expression in the host (ptp-F1/ptp-R1).

Acknowledgements

The authors gratefully acknowledge USDA NRI grant 99-35302-8522 for supporting this study.

References

Asgari, S., Hellers, M. and Schmidt, O. (1996) Host hemocyte inactivation by an insect parasitoid: Transient expression of a polydnavirus gene. *J General Virol* **77**: 2653–2662.

Beckage, N.E. (1985) Endocrine interactions between endoparasitic insects and their hosts. *Ann Rev Entomol* **30**: 371–413.

Beckage, N.E., Tan, F.F., Schleifer, K.W., Lane, R.D. and Cherubin, L.L. (1994) Characterization and biological effects of *Cotesia congregata* polydnavirus on host larvae of the tobacco hornworm, *Manduca sexta*. *Arc Insect Biochem Physiol* **26**: 165–195.

Bell, R.A., Owens, C.D., Shapiro, M. and Tardif, J.R. (1981) Development of mass-rearing technology. In *The Gypsy Moth: Research Toward Integrated Pest Management* (Donae, C.C. and McManus, M.L., eds), pp. 599–633. USDA Tech. Bull. #1584.

Blissard, G.W., Fleming, J.G.W., Vinson, S.B. and Summers, M.D. (1986a) *Campoletis sonorensis* virus: Expression in *Heliothis virescens* and identification of expressed sequences. *J Insect Physiol* **31**: 351–359.

Blissard, G.W., Vinson, S.B. and Summers, M.D. (1986b) Identification, mapping and *in vitro* translation of *Campoletis sonorensis* virus mRNAs from parasitized *Heliothis virescens* larvae. *J Virol* **57**: 318–327.

Cui, L. and Webb, B.A. (1996) Isolation and characterization of a member of the cysteine-rich gene family from *Campoletis sonorensis* polydnavirus. *J General Virol* **77**: 797–809.

Cui, L. and Webb, B.A. (1997) Homologous sequences in the *Campoletis sonorensis* polydnavirus genome are implicated in replication and nesting of the W segment family. *J Virol* **71**: 8504–8513.

Davies, D.H., Strand, M.R. and Vinson, S.B. (1987) Changes in differential haemocyte count and *in vitro* behavior of plasmatocytes from host *Heliothis virescens* caused by *Campoletis sonorensis* polydnavirus. *J Insect Physiol* **33**: 143–153.

Dib-Hajj, S.D., Webb, B.A. and Summers, M.D. (1993) Structure and evolutionary implications of a 'cysteine-rich' *Campoletis sonorensis* polydnavirus gene family. *Proc Natl Acad Sci USA* **90**: 3765–3770.

Dover, B.A., Davies, D.H., Strand, M.R., Gray, R.S., Keeley, L.L. and Vinson, S.B. (1987) Ecdysteroid titer reduction and developmental arrest of last instar *Heliothis virescens* larvae by calyx fluid from parasitoid *Campoletis sonorensis*. *J Insect Physiol* **33**: 333–338.

Dover, B.A., Davies, D.H. and Vuinson, S.B. (1988) Degeneration of last instar *Heliothis virescent* prothoracic glands by *Campoletis sonorensis* polydnavirus. *J Invertebrate Pathol* **51**: 80–91.

Dushay, M.S. and Beckage, N.E. (1993) Dose-dependent separation of *Cotesia congregata*-associated polydnavirus effects on *Manduca sexta* larval development and immunity. *J Insect Physiol* **39**: 1029–1040.

Edson, K.M., Vinson, S.B., Stoltz, D.B. and Summers, M.D. (1981) Virus in a parasitoid wasp: Suppression of the cellular immune response in the parasitoid's host. *Science* **211**: 582–583.

Fleming, J.G.W., Blissard, G.W., Summers, M.D. and Vinson, S.B. (1983) Expression of *Campoletis sonorensis* virus in the parasitized host *Heliothis virescens*. *J Virol* **48**: 74–78.

Gundersen-Rindal, D. and Dougherty, E.M. (2000) Evidence for integration of *Glyptapanteles indiensis* polydnavirus DNA into the chromosome of *Lymantria dispar* in vitro. *Virus Res* **66**: 27–37.

Harwood, S.H., Grosovsky, A.J., Cowles, E.A., Davis, J.W. and Beckage, N.E. (1994) An abundantly expressed hemolymph glycoprotein isolated from newly parasitized *Manduca sexta* larvae is a polydnavirus gene product. *Virology* **205**: 381–392.

Johner, A. and Lanzrein, B. (2002) Characterization of two genes of the polydnavirus of Chelonus inanitus and their stage-specific expression in the host *Spodoptera littoralis*. *J General Virol* **83**: 1075–1085.

Lavine, M.D. and Beckage, N.E. (1996) Temporal pattern of parasitism-induced immunosupressive in *Manduca sexta* larvae parasitized by *Cotesia congregata*. *J Insect Physiol* **42**: 39–49.

Li, X. and Webb, B.A. (1994) Apparent functional role for a cysteine-rich polydnavirus protein in suppression of the insect cellular immune response. *J Virol* **68**: 7482–7489.

Luckhart, S. and Webb, B.A. (1996) Interaction of a wasp ovarian protein and polydnavirus in host immune suppression. *Dev Comp Immun* **20**: 1–21.

Menzo, S.P., Bannarelli, M., Giacca, A., Manzin, P.E., Varaldo, E. and Clementi, M. (1992) Absolute quantitation of viremia in human immunodeficiency virus infection by competitive reverse transcription and polymerase chain reaction. *J Clin Microbio* **30**: 1752–1757.

Pennacchio, F., Falabella, P., Sordetti, R., Varricchio, P., Malva, C. and Vinson, S.B. (1998a) Prothoracic gland inactivation in *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) larvae parasitized by *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae). *J Insect Physiol* **44**: 845–857.

Pennacchio, F., Falabella, P. and Vinson, S.B. (1998b) Regulation of *Heliothis virescens* prothoracic glands by *Cardiochiles nigriceps* polydnavirus. *Arch Insect Biochem Phys* **38**: 1–10.

Strand, M.R., Mackenzie, D.I., Grassl, V., Dover, B.A. and Aiken, J.M. (1992) Persistence and expression of *Microplitis demolitor* polydnavirus in *Pseudoplusia includens*. *J General Virol* **73**: 1627–1635.

Strand, M.R. and Pech, L.L. (1995) *Microplitis demolitor* polydnavirus induces apoptosis of a specific hemocytes morphotype in *Pseudoplusia includens*. *J General Virol* **76**: 283–291.

Strand, M.R., Witherell, A.R. and Trudeau, D. (1997) Two *Mircoplitis demolitor* polydnavirus mRNAs expressed in hemocytes of *Pseudoplusia includens* contain a common cysteine-rich domain. *J Virol* **71**: 2146–2156.

Summers, M.D. and Dib-Hajj, S.D. (1995) Polydnavirus-facilitated endoparasite protection against host immune defenses. *Proc Nat Acad Sci USA* **96**: 29–36.

Tanaka, T., Agui, N. and Hiruma, K. (1987) The parasitoid *Apanteles kariyai* inhibits pupation of its host, *Pseudaletia separate*, via disruption of prothoracicotropic hormone release. *Gen Compara Endocrinol* **67**: 364–374.

Tanaka, T. and Vinson, S.B. (1991) Depression of prothoracic gland activity of *Heliothis virescens* by venom and calyx fluids from parasitoid, *Cardiochiles nigriceps*. *J Insect Physiol* **37**: 139–144.

Theilmann, D.A. and Summers, M.D. (1986) Molecular analysis of *Campoletis sonorensis* virus DNA in the lepidopteran host *Heliothis virescens*. *J Gen Virol* **67**: 1961–1969.

Theilmann, D.A. and Summers, M.D. (1988) Identification and comparison of the *Campoletis sonorensis* virus transcripts expressed from four genomic segments in the insect hosts *Campoletis sonorensis* and *Heliothis virescens*. *Virology* **167**: 329–341.

Vinson, S.B., Edson, K.M. and Stoltz, D.B. (1979) Effect of a virus associated with the reproduction system of the parasitoid wasp, *Campoletis sonorensis*, on host weight gain. *J Invertebrate Pathol* **34**: 133–137.

Xu, D. and Stoltz, D.B. (1993) Polydnavirus genome segment families in the ichneumonid parasitoid *Hyposoter fugitivus*. *J Virol* **67**: 1340–1349.

Yamanaka, A., Hayakawa, Y., Noda, H., Nakashima, N. and Watanabe, H. (1996) Characterization of polydnavirus-encoded RNA in parasitized armyworm larvae. *Insect Biochem Mol Biol* **5**: 529–536.

Zimmermann, K. and Mannhalter, J.W. (1996) Technical aspects of quantitative competitive PCR. *Biotechniques* **21**: 268–279.